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Protective effects of gallic acid against chronic cerebral hypoperfusion-induced cognitive deficit and brain oxidative damage in rats

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ABSTRACT

Free radical-induced neural damage is implicated in cerebral hypoperfusion disorders and antioxidants have protective effects. In the present study, we examined the effect of gallic acid (GA; 100 mg/kg, p.o. for 10 days) on cognitive deficit and cerebral oxidative stress induced by permanent bilateral common carotid artery occlusion (2VO) as an animal model of vascular dementia (VD). The results showed that 2VO significantly reduced the spatial memory performance in Morris water maze as well as non-enzymatic (total thiol) and enzymatic [glutathione peroxidase (GPx)] antioxidant contents and increased the level of malondialdehyde (MDA) in the hippocampus and frontal cortex of vehicle-treated group as compared to sham-operated rats. Furthermore, chronic administration of GA significantly restored the spatial memory, total thiol and GPx contents and also decreased MDA levels in these tissues. GA alone did not show any change neither in the status of various antioxidants nor behavioral tests over sham values. The results demonstrate that GA has beneficial activity against 2VO-induced cognitive deficits *via* enhancement of cerebral antioxidant defense. Taken together, the present study suggested that GA might be useful in the treatment of VD.

1. Introduction

Ischemic brain injury is caused by the interruption of cerebral blood flow leading to excessive production of reactive oxygen species which is associated with behavioral dysfunction and also cognitive deficits (Li et al., 2011). The formation of reactive oxygen species represents the first key step to initiate tissue oxidative stress (Silva-Adaya et al., 2008). Oxidative stress is defined as an imbalance between cellular levels of reactive oxygen species (*e.g.*, superoxide and hydroxyl radicals) and cellular antioxidant defense. Reactive oxygen species are produced by a free radical chain reaction caused tissue injury by reacting with biomolecules such as lipids, proteins, and nucleic acids as well as by depleting enzymatic and/or non-enzymatic antioxidants in the brain. In order to scavenge reactive oxygen species, various defense systems such as glutathione, antioxidant enzymes [glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase (CAT)] exist in the brain (Akdag et al., 2010).

Gallic acid (GA, 3,4,5-trihydroxybenzoic acid), is one of the most important polyphenolic substances in plants which is present in grapes, different berries, mango, areca nut, walnut, green tea and other fruits as well as in wine. Also, it is considered as putative active compound in tannin, namely gallotannin (Singh et al., 2004). This compound possesses antioxidant, free radical scavenging, anti-cancer, anti-inflammatory (Bhouri et al., 2012), anti-fungal (Choi et al., 2010) and anti-human rhinovirus activities (Nguyen et al., 2013). Gallic acid has also been used in food, cosmetics, and in pharmaceuticals as an antioxidant (Bhouri et al., 2012). GA can be used to treat human albuminuria and diabetes (Hsieh et al., 2007). Moreover, it has been reported that GA produced anti-anxiety (Dhingra et al., 2012), anti-depressant (Chhillar and Dhingra, 2013) and anti-epileptic (Huang et al., 2012) effects in animal models.

Due to the free radical scavenging property, GA-containing plant extracts have showed anti-diabetic, anti-angiogenic and anti-melanogenic effects and reduced heart infarction incidence and the oxidative damage of liver and kidney tissues (Priscilla

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and Prince, 2009). It has been reported that GA is involved in the protection of neural cells against *in vitro* β-amyloid peptide (Aβ)-induced death (Ban et al., 2008). Recently, our research indicated the neuroprotective effect of GA against cerebral oxidative stress induced by 6-hydroxydopamine and or streptozotocin in rat brain (Mansouri et al., 2013a,b). In addition, Ferruzzi et al. (2009) demonstrated that repeated treatment of rodents with grape seed extract significantly increased the bioavailability and brain deposition of GA which previously found to attenuate cognitive deterioration in a mouse model of Alzheimer's disease. Thus, GA as a major constituent of grape seed extract may be a potential neuroprotective agent.

To the best of our knowledge, there is no published scientific report on the protective role of GA against cognitive deficits and cerebral oxidative stress induced by chronic cerebral hypoperfusion. Therefore, the present study intended to examine the effects of GA on learning and memory impairments induced by permanent cerebral artery occlusion (2VO) and determined whether this effect was modulated through antioxidant mechanisms in the brain.

2. Materials and methods

2.1. Chemicals

DTNB (2,2′-dinitro-5,5′-dithiodibenzoic acid), TBA (2-thiobarbituric acid), *n*-butanol, tris base, ethylendiamine tetraacetic acid disodium, sodium acetate, glacial acetic acid, phosphoric acid, potassium chloride, tetramethoxypropane were obtained from Merck Company (Darmstadt, Germany) and GA was purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade and prepared from Merck Company (Darmstadt, Germany).

2.2. Animals

Adult male Wistar rats weighing 200–250 g were used throughout the study. All animals were obtained from the Animal House of Shahrekord Medical School (Shahrekord, Iran). Animals were allowed free access to standard laboratory chow and water, *ad libitum*. A 12-h light/dark cycle at 22 ± 2 °C and 50% humidity conditions were maintained. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals adopted by National Institute of Health and the Federation of Iranian Societies for Experimental Biology.

2.3. Surgery

Permanent cerebral hypoperfusion (PCH), that mimics the vascular dementia model, was induced by occlusion of the bilateral common carotid arteries (two vessels occlusion; 2VO) (Xu et al., 2012). Briefly, a neck ventral midline incision was made and the common carotid arteries were then exposed and gently separated from the vague nerve. The bilateral common carotid arteries were tied with silk threads, whilst the rats were under an appropriate level of ketamine/xylazine (50/5 mg/kg) anesthesia (Roohbakhsh et al., 2007).

2.4. Experimental design

The animals were randomly divided into 4 groups (seven each). Group 1 was the sham-operated (sham) in which normal saline (2 ml/kg) was given by oral gavage. Group 2 was 2VO in which bilateral common carotid artery was ligated and received normal saline as the same of sham group. Group 3 was sham + GA which treated with GA at dose of 100 mg/kg (Hsu and Yen, 2007). Group 4 was 2VO + GA in which bilateral common carotid artery was ligated and received GA at dose of 100 mg/kg. GA administration was started 5 days before surgery and continued for 10 consecutive days.

2.5. Assessment of spatial memory via Morris water maze test

The spatial memory performance was evaluated using a Morris water maze (MWM). The water maze used was a black circular tank (136 × 60, diameter × height) which filled with water (20 ± 1 °C) to a depth of 25 cm. The maze was located in a room containing extra-maze cues (posters). The pool geographically divided into four quadrants [northeast (NE), northwest (NW), southeast (SE), southwest (SW)] and starting positions [north (N), south (S), east (E), west (W)] that were equally spaced around the perimeter of the pool. A hidden circular platform (diameter: 13 cm) was located 2 cm below the surface of the water on a fixed location in one of the four quadrants of the pool. A video Camera was mounted directly above the water maze to record the rats' swim paths. An automated tracking system (EthoVision®, Noldus, Wageningen, Netherlands) was used to measure the escape latency and percentage of the time in the target quadrant. 6 days after 2VO (Kim et al., 2006), rats were given four training trials each day for 4 consecutive days. For each training trial, the rats were placed in the water facing the pool wall at one of the four starting in a different order each day and allowed to swim until they reached the platform. The latency to reach the platform was recorded for up to 60 s. The rats remaining on the platform for 30 s were removed. One day after the last training, a probe trial was conducted by removing the platform. Rats were allowed to swim freely into the pool for 60 s. The time spent in the target quadrant, which had previously contained the hidden platform, was recorded. The time spent in the target quadrant indicated the degree of memory consolidation that has taken place after learning. After the trials, the rats were dried with a towel and placed in a holding cage under a heating lamp before returning to the home cage. A visible platform trial was performed with the platform placed on the side of the pool opposite its location during hidden platform training to check the vision of all rats (Itoh et al., 1999; Morris, 1984). In order to determine whether the group differences in escape latency and swimming distance were due to their differences in swimming ability, the swimming speed was also evaluated.

2.6. Brain sample collection and biochemical assays

At the end of behavioral experiments, the animals were decapitated and the hippocampus and frontal cortex were removed quickly, rinsed with saline, and then frozen in a freezer (−80 °C) until used. The tissues were homogenized in cold KCl solution (1.5%) to give a 10% homogenate suspension used for measuring thiobarbituric acid reactive substances value (expressed as malondialdehyde (MDA) equivalents), total thiol contents and GSH-Px activity (Naghizadeh et al., 2008).

2.6.1. Thiobarbituric acid reactive species measurement

MDA levels, an index of lipid peroxidation (LPO), produced by free radicals were measured. MDA reacts with thiobarbituric acid to produce a red colored complex that has peak absorbance at 532 nm. Briefly, 3 ml phosphoric acid (1%) and 1 ml TBA (0.6%) were added to 0.5 ml of homogenate in a centrifuge tube and the mixture was heated for 45 min in a boiling water bath. After cooling, 4 ml *n*-butanol was added to the mixture and vortex-mixed for 1 min followed by centrifugation at 2000 rpm for 20 min. The colored layer was transferred to a fresh tube and its absorbance was measured at 532 nm. MDA levels were

determined using 1,1,3,3-tetramethoxypropane as standard. The standard curve of MDA was constructed over the concentration range of 0–10 μM (Naghizadeh et al., 2008).

2.6.2. Determination of glutathione peroxidase concentration

GSH-Px concentration was measured with the GSH peroxidase kit (Randox Company, England) (Abdel-Salam et al., 2012).

2.6.3. Total thiol (–SH) groups assay

Total –SH groups were measured using DTNB (5,5′-dithiobis-2-nitrobenzoic acid) as the reagent (Kamboj et al., 2008). This reagent reacts with the –SH groups to produce a yellow colored complex which has a peak absorbance at 412 nm. Briefly, 1 ml tris–EDTA buffer (pH 8.6) was added to 50 μl brain homogenate in 2 ml cuvettes and absorbance was read at 412 nm against tris–EDTA buffer alone (A1). Then, 20 μl DTNB reagents (10 mM in methanol) was added to the mixture and after 15 min (stored in laboratory temperature), the sample absorbance was read again (A2). The absorbance of DTNB reagent was also read as a blank (B). Total thiol concentration (mM) was calculated from the following equation:

$$\text{Total thiol concentration (mM)} = \frac{(A2 - A1 - B) \times 1.07}{0.05 \times 13.6}$$

2.7. Statistical analysis

Results were expressed as mean ± S.E.M. The behavioral data of learning in MWM test was analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni's post test. The biochemical data and memory performance in MWM test were analyzed by one-way ANOVA followed by Tukey's test. All analysis was performed using the Prism 5.0 (San Diego, CA, USA) statistical package program. Statistical significance was ascertained at $P < 0.05$.

3. Results

3.1. Effects of GA against 2VO-induced learning and memory impairment in MWM test

Fig. 1a shows the reduction of latency time to find the hidden platform in all groups during the four-day training trials in MWM task. Analysis or the results revealed that 2VO animals presented a higher latency time than sham group ($P < 0.001$), showing poorer learning performance due to PCH. But, chronic treatment with GA significantly improved the learning performance in 2VO+GA group as compared to 2VO animals ($P < 0.01$). Moreover, administration of GA *per se* in sham+GA group did not modify basal learning performance of rats during 4 days of training as compared to sham group ($P > 0.05$).

According to Fig. 1b, on the probe trial day with the platform removed, rats in the 2VO+GA group failed to remember the precise location of platform, spending less time in the target quadrant than sham group ($P < 0.001$). But, the time spending in the target quadrant was significantly increased with chronic administration of GA as compared to 2VO group indicating improved consolidation of memory ($P < 0.001$). Moreover, administration of GA *per se* in sham+GA group did not affect the parameter as compared to sham group ($P > 0.05$). Furthermore, there was no significant difference among the swim speeds of all four groups (Fig. 1c, $P > 0.05$). This excludes the possibility that the activity *per se* may have contributed to the severe deficits in MWM after 2VO surgery.

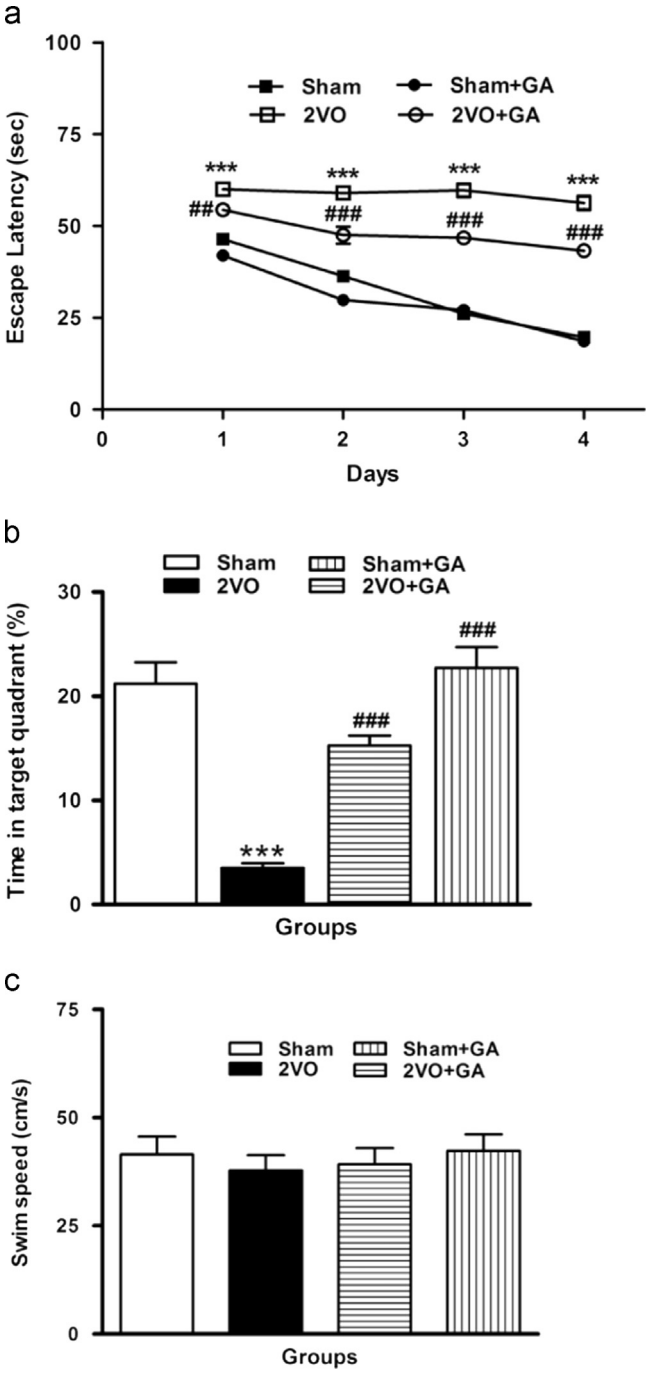

Fig. 1. Effects of gallic acid (GA, 100 mg/kg/day, p.o., 10 days) on (a) escape latency to find the hidden platform, (b) percentage of time spent in target quadrant during the probe trials and (c) swim speed in the Morris water maze test in 2-vessle occlusion (2VO) rats. Data are expressed as mean ± S.E.M. ($n=7$). $^{***}P < 0.05$; 2VO compared to sham group from first day of the training sessions; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, 2VO+GA compared to 2VO group from first day of the training sessions.

3.2. Effect of GA on MDA levels in the hippocampus and frontal cortex

The degree of free radical damage following 2VO was assessed using LPO, which measured as MDA levels. According to Fig. 2, there was an increase in MDA levels in the hippocampus and frontal cortex of 2VO group ($P < 0.001$) as compared to sham group. Oral administration of GA resulted in a significant reduction of MDA levels in the brain tissues of 2VO+GA animals as

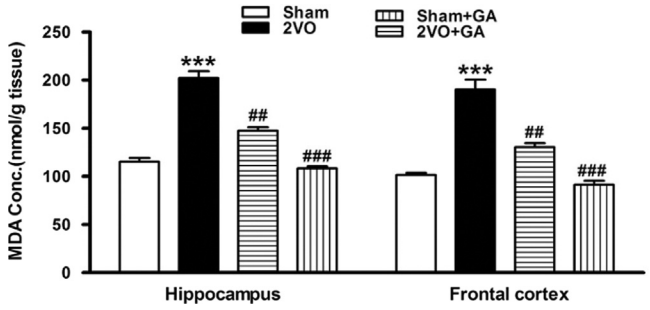

Fig. 2. Effect of GA on MDA levels in the hippocampus and frontal cortex of 2-vessle occlusion (2VO) rats. Values are expressed as mean ± S.E.M ($n=7$). $^{***}P<0.001$ as compared to sham group. $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$ as compared to 2VO group.

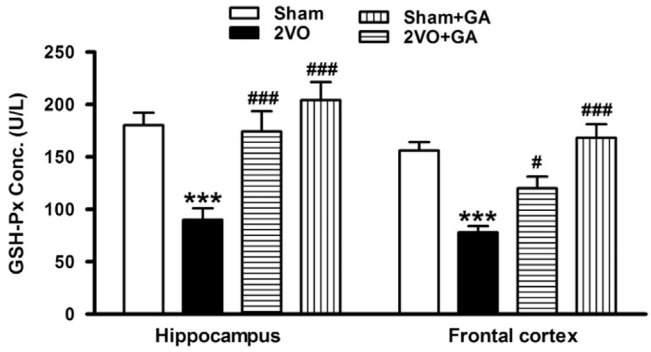

Fig. 3. Effect of GA on GSH-Px concentration in the hippocampus and frontal cortex of 2-vessle occlusion (2VO) rats. Values are expressed as mean ± S.E.M ($n=7$). $^{**}P<0.01$, $^{***}P<0.001$ as compared to sham group. $^{\#}P<0.05$; $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$ as compared to 2VO group.

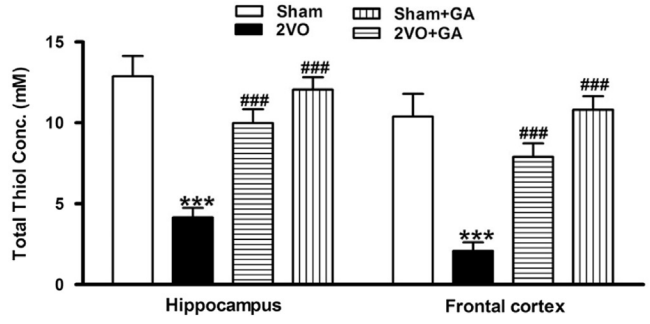

Fig. 4. Effect of GA on total thiol concentration in the hippocampus and frontal cortex of 2-vessle occlusion (2VO) rats. Values are expressed as mean ± S.E.M ($n=7$). $^{***}P<0.001$ as compared to sham group; $^{\#}P<0.05$; $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$ as compared to 2VO group.

compared to 2VO group ($P<0.001$). GA *per se* did not influence the MDA levels.

3.3. Effect of GA on GSH-Px activity in the hippocampus and frontal cortex

GSH-Px concentration (μ/l) was measured to evaluate the enzymatic defense potential of the cells against the oxidative stress. According to Fig. 3, the GSH-Px concentration was significantly decreased in 2VO group as compared to sham group in both hippocampus and frontal cortex ($P<0.01$, $P<0.001$; respectively). However, the decrease of the GSH-Px concentration was significantly restored by GA treatment in the brain tissues of 2VO+GA group ($P<0.01$, $P<0.001$; respectively). GA *per se* did not affect the GSH-Px concentration.

3.4. Effect of GA on total thiol levels in the hippocampus and frontal cortex

The total thiol concentration (mM) was measured to evaluate the non-enzymatic defensive potential of the cell against oxidative stress. According to Fig. 4, total thiol contents in 2VO animals were found to be significantly depleted as compared to sham group in the hippocampus and the frontal cortex ($P<0.001$). Treatment with GA in 2VO+GA group was able to raise total thiol contents significantly as compared to 2VO animals ($P<0.01$). GA *per se* did not affect the total thiol levels.

4. Discussion

The present study intended to evaluate the neuroprotective effect of gallic acid (GA), a natural polyphenolic compound, on the permanent cerebral hypoperfusion (PCH) induced cognitive and biochemical alterations in rats. PCH produced a marked impairment in cognitive function, which was associated with significant increase in the oxidative stress in rat brain (Peng et al., 2007). However, chronic treatment with GA significantly alleviated the cognitive dysfunction and restored oxidative stress markers in cerebral hypoperfused rats.

The permanent occlusion of bilateral common carotid arteries in rats is a common cerebral ischemic model to investigate vascular dementia and drug effect on this disorder (Wang et al., 2000). Oxidative stress has been suggested to play a key role in hypoxic- ischemic brain damage and also correlates with expression of inflammatory proteins in the cerebral vasculature and impairment of cognitive function (Evola et al., 2010). Reactive oxygen species can oxidize membrane lipids, essential cellular proteins, and DNA that cause cellular injury. Free radicals catalyze the peroxidation of unsaturated fatty acids in cell membranes; thus, generating MDA, a lipid peroxidation marker (Bilenko et al., 1988; Akdag et al., 2010). In the present study, compared to those in sham group, the MDA levels in GA-treated group significantly decreased, whereas the GSH and GSH-Px contents were preserved. These findings are consistent with earlier reports showing that GA might attenuate the excessive formation of reactive oxygen species and compensate antioxidant ability secondary to oxidative insults. It has been reported that GA exerted the protective effects against amyloid β-mediated neurotoxicity (Bastianetto et al., 2006), lead nitrate, streptozotocin, kainic acid and or 6-OHDA induced brain oxidative damages (Prince et al., 2011; Reckziegel et al., 2011; Huang et al., 2012; Mansouri et al., 2013a,b). Li et al. (2005) showed that when GA was used to treat the 9-month-old male senescence accelerated mice not only reinstated the activities of CAT and GSH-Px but also significantly reduced the amount of MDA in the brain, liver and kidney. Also, pre-treatment of SH-SY5Y human cells with GA and its derivatives suppressed 6-OHDA induced oxidative stress *in vitro* to various degrees (Lu et al., 2006). In addition to protective effects in neurotoxicity models, GA counteracted the oxidative damage caused by lindane and carbon tetrachloride in rat liver and kidney (Jadon et al., 2007; Padma et al., 2011). Other researchers have also reported that antioxidants could protect against cognitive deficits induced by chronic cerebral hypoperfusion. Azzubaidi et al. (2012) showed the protective effect of black cumin seed on learning ability and memory in a rat model of chronic cerebral hypoperfusion. Chrysin (5,7-dihydroxyflavone), a flavonoid in *Passiflora caerulea*, has therapeutic potential for the treatment of neurodegeneration and dementia caused by decreased cerebral blood flow (He et al., 2012). In another study, *Z*-ligustilide significantly prevented hypoperfused cognitive deficits and brain damage through an antioxidant effect and by improvement of cholinergic activity (Kuang et al., 2008). As shown

in a study by Xu et al. (2010), green tea polyphenols, which are potent antioxidants and free radical scavengers, can attenuate vascular cognitive impairment. The ethanolic extract of saffron and its active constituent, crocin, have also shown to exert protective effect against PCH in rats (Hosseinzadeh et al., 2012). These studies indicate that the antioxidant effects of these compounds play an important role in improving the spatial cognitive abilities after PCH.

Previous studies have demonstrated that central cholinergic system is damaged by PCH in rats (Tanaka et al., 1996; Ni et al., 1995) and stimulation of the above system improves spatial memory deficits in this animal model (Murakami et al., 1997). Also, it has been reported that glutamate release in the brain was increased following cerebral ischemia (Davalos et al., 2000). This neurotransmitter is capable to generate reactive oxygen species and induction of neural toxicity and death (Novelli et al., 1988). Moreover, it is well-established that some drugs can improve the learning and memory of patients with Alzheimer's disease or VD by modulating classic neurotransmitters such as glutamate and acetylcholine. Also, various medications such as antioxidants, radical scavengers and calcium antagonists have been approved for the treatment of VD around the world (Itil et al., 1998). It has been demonstrated that GA can inhibit the elevation of glutamate release, Ca^{2+} and reactive oxygen species generation and also has anticholinesterase activity in the brain tissue. These effects of GA could be associated with its neuroprotective properties (Ban et al., 2008; Ghayur et al., 2011; Hong et al., 2012).

In conclusion, our data demonstrated that treatment with GA could improve the cognitive deficits and 2VO-induced cerebral damages in rats. Cognitive enhancing and neuroprotective effect of GA are associated with the anti-oxidant and partly at least anti-cholinesterase and glutamate inhibiting activities of this compound. However, the present results do not exclude the other mechanisms involved in the gallic acid action. Further preclinical studies are necessary to elucidate the mechanism(s) of action of this compound including modulation of the cholinergic and glutamatergic neurotransmission. Altogether, these findings may provide pharmacological basis for the use of GA as an antioxidant metabolite of ellagitannins in the treatment and prevention of neurodegenerative diseases such as vascular dementia and could explain the protective effects of ellagitannin consumed from natural sources.

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